

Serial No. 09/431,888

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Applicant: LYN M. WISE ET AL.

Serial No.: 09/431,888 Group Art Unit: 1646

Filed: NOVEMBER 2, 1999 Examiner: Andres, J.

Title: VASCULAR ENDOTHELIAL GROWTH FACTOR-LIKE  
PROTEIN FROM ORF VIRUS NZ2 BINDS AND ACTIVATES  
MAMMALIAN VEGF RECEPTOR-2, AND USES THEREOFDECLARATION UNDER 37 C.F.R. § 1.132Commissioner for Patents  
Washington, D.C. 20231

Sir:

I, Andrew A. Mercer, hereby declare that:

1. I am a citizen of New Zealand and my mailing address is Virus Research Unit, Department of Microbiology, University of Otago, P.O. Box 56, Dunedin, New Zealand.

2. I was educated at the University of Otago, Dunedin, New Zealand where I received my Ph.D. in Microbiology in 1979, my Postgraduate Diploma in Science for Microbiology in 1974, and my Bachelor of Science Degree in Microbiology in 1973.

3. Since 1993, I have been an MRC Senior Research Fellow and the Director of the Virus Research Unit of the Department of Microbiology at the University of Otago. Prior to my acceptance of this position, from 1984 to 1992, I was a Research Officer at the MRC Virus Research Unit in Dunedin, New Zealand. While at the MRC Virus Research Unit, I was also a visiting scientist at INSERM, Université Louis Pasteur, Strasbourg, France in 1990 and at the Moredun Research Institute, Edinburgh, Scotland in 1991. In 1993, I was a postdoctoral fellow at the Department of Microbiology,

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University of Otago and from 1980-1982, I was postdoctoral fellow at the Max Planck Institute for Molecular Genetics in Berlin, Federal Republic of Germany. Prior to that, from 1978 to 1979, I was a scientific officer at the Department of Microbiology at the University of Otago.

4. Over the past ten years, I have been a co-author in 34 scientific publications which have reported the findings of my experimental collaborations.

5. I am one of the named inventors in the above-identified U.S. Patent Application, and I am making this Declaration in support of said patent application.

6. The presently claimed invention is directed to the specificity of two VEGF-like proteins, ORFV2-VEGF and NZ10, for VEGFR-2 and the uses resulting from the activation of VEGFR-2 by the ORFV2-VEGF and NZ10.

7. In the developing embryo, the primary vascular network is established by *in situ* differentiation of mesodermal cells in a process called vasculogenesis. The sprouting of new capillaries from the pre-existing vasculature is a process called angiogenesis. The family of vascular endothelial growth factors (VEGFs), and their corresponding receptors is primarily responsible for stimulation of endothelial cell growth and differentiation and for certain functions of the differentiated cells. VEGF has important regulatory functions in the formation of new blood vessels during embryonic vasculogenesis and in angiogenesis during adult life. Carmeliet et al., Nature 380: 435-439 (1996). The significance of the role of VEGF has been demonstrated by *in situ* studies showing that inactivation of a single VEGF allele results in embryonic lethality due to failed development of the vasculature. Carmeliet et al., Nature 380: 435-439 (1996); Ferrara et al., Nature 380: 439-442 (1996).

8. The VEGFs are members of the PDGF family and act primarily via endothelial receptor tyrosine kinase receptors. The only receptor tyrosine kinases known to bind VEGFs are VEGFR-1, VEGFR-2, and VEGFR-3. Disruption of the VEGFR genes results in aberrant development of the vasculature leading to

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embryonic lethality around midgestation. Analysis of homozygous mice with inactivated alleles of VEGFR-1 suggests that this receptor is required for functional organization of the endothelium. Analysis of homozygous mice with inactivated alleles of VEGFR-2 suggests that this receptor is required for endothelial cell proliferation, hematopoiesis, and vasculogenesis. Analysis of homozygous mice with inactivated alleles of VEGFR-3 suggests that this receptor is required for organization of large vessels such as cardiac vessels.

9. In addition to the foregoing, VEGF-like proteins have been identified which are encoded by four different strains of the orf virus; the orf virus is the first virus reported to encode a VEGF-like protein. The first two strains are NZ2 and NZ7, described in Lyttle et al., J. Virol. 68: 84-86 (1994); the third is P1701 described in Meyer et al., The EMBO Journal 18:363-374 (1999); and the fourth strain is NZ10 described in the instant application. Because these proteins show amino acid sequence similarity to VEGF and to each other, these VEGF-like proteins are being studied to see if they are capable of inducing characteristic VEGF-like effects such as mitogenesis of mesodermal and endothelial cells and vascular permeability.

10. The claimed subject matter is based on the discovery that the VEGF-like protein encoded by NZ2 (hereinafter referred to as ORFV2-VEGF) and NZ10 are capable of binding to the extracellular domain of VEGFR-2 to form bioactive complexes which mediate useful cellular responses or antagonize undesired biological activities.

11. Example 5 of the instant specification demonstrates the mitogenicity of ORFV2-VEGF for endothelial cells. In that example, the mitogenic capacity of ORFV2-VEGF was tested using human umbilical vein endothelial cells (HUVECs). Washed and trypsinized HUVECs were aliquoted at  $10^3$  cells per well in a 96 well plate and allowed to adhere for 6-16 hours at 37°C in EBM-2 medium plus serum and no growth factors. The growth factors indicated in Figure 6, ORFV2-VEGF, VEGF-164, and VEGF-DANAC, were then added to the cells for 3 days at 37°C, trypsinized

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and counted. The amount of cellular proliferation was quantified by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay which measures the conversion of the MTT substrate. As figure 6 indicates, 0.5-1.0 ng/mL of ORFV2-VEGF was able to stimulate an increase in the number of cells after three days; the proliferation index at 10 ng/mL of ORFV2-VEGF being approximately 0.55.

12. Attached to this declaration are two other experiments which demonstrate the mitogenic activity of both ORFV2-VEGF and NZ10. These experiments are conducted with the same procedure set forth in Example 5 except that human microvascular endothelial cells (HMVECs) are used in place of HUVECs and the amount of cellular proliferation was quantified by direct counting of cell numbers. Figure 6A shows that 0.5 ng/mL of NZ10 results in a proliferation index of approximately 0.5 and that the mitogenicity of NZ10 peaks at 125 ng/mL of NZ10 as demonstrated by a proliferation index of approximately 2.0. For ORFV2-VEGF, 0.5 ng/mL results in a proliferation index of approximately 0.75 with the mitogenic peak occurring between 125 and 500 ng/mL where a proliferation index of approximately 2.5 is recorded. Figure 6B reproduces the results shown in Figure 6A with a greater range of molecules for comparison. These experiments clearly show that the mitogenic properties of NZ10 with respect to endothelial cells are comparable to those of ORFV2-VEGF. Both ORFV2-VEGF and NZ10 show the initiation of a mitogenic response at concentration of 0.5ng/mL with peak mitogenicity occurring at or about 125 ng/mL.

13. Example 2 of the instant specification describes the bioassay for ORFV2-VEGF/NZ10 binding to VEGFR-2. Figure 3 shows the results of these experiments for ORFV2-VEGF and not for NZ10. In Example 2,  $10^4$  IL-3 conditioned Ba/F3 cells expressing the VEGFR-2-EpoR receptor were washed in IL-3 medium and aliquoted into a 96 well plate and incubated for 40 hours at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The bioassay cells can proliferate in the absence of IL-3 when the chimeric receptor is activated. Cell proliferation was

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quantified by counting the degree of incorporation of  $^3\text{H}$ -thymidine which was added 4 hours prior to cell harvesting. Figure 3 shows that ORFV2-VEGF was able to induce detectable DNA synthesis in the bioassay cell line at a concentration of 25 ng/mL and VEGF induced DNA synthesis in the bioassay cell line from a concentration of 5 ng/mL. Figure 3A attached to this declaration shows that NZ10 was also able to induce DNA synthesis in the bioassay cell line at a concentration of 25 ng/mL with a potency slightly reduced to that of ORFV2-VEGF and VEGF-DANAC. The results of these experiments show that both ORFV2-VEGF and NZ10 can bind to and cross-link the extracellular domain of VEGFR-2 and induce a proliferative response.

14. The results of Example 2 and Figures 3 and 3A show that ORFV2-VEGF and NZ10 both bind to VEGFR-2 and induce a proliferative response. Example 5 and Figures 6, 6A and 6B show that both ORFV2-VEGF and NZ10 activate VEGFR-2 to induce endothelial cell mitogenesis. The ability of these VEGF-like proteins to stimulate DNA synthesis and endothelial cell proliferation demonstrates that ORFV2-VEGF and NZ10 act as agonists and not antagonists of VEGFR-2. In Figure 6B, The proliferation data for VEGF from pseudocowpox virus (PCPV) was included in these experiments as an additional control.

15. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that those statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date:

18/4/01  
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Andrew A. Mercer

## VEGFR-2 Bioassay

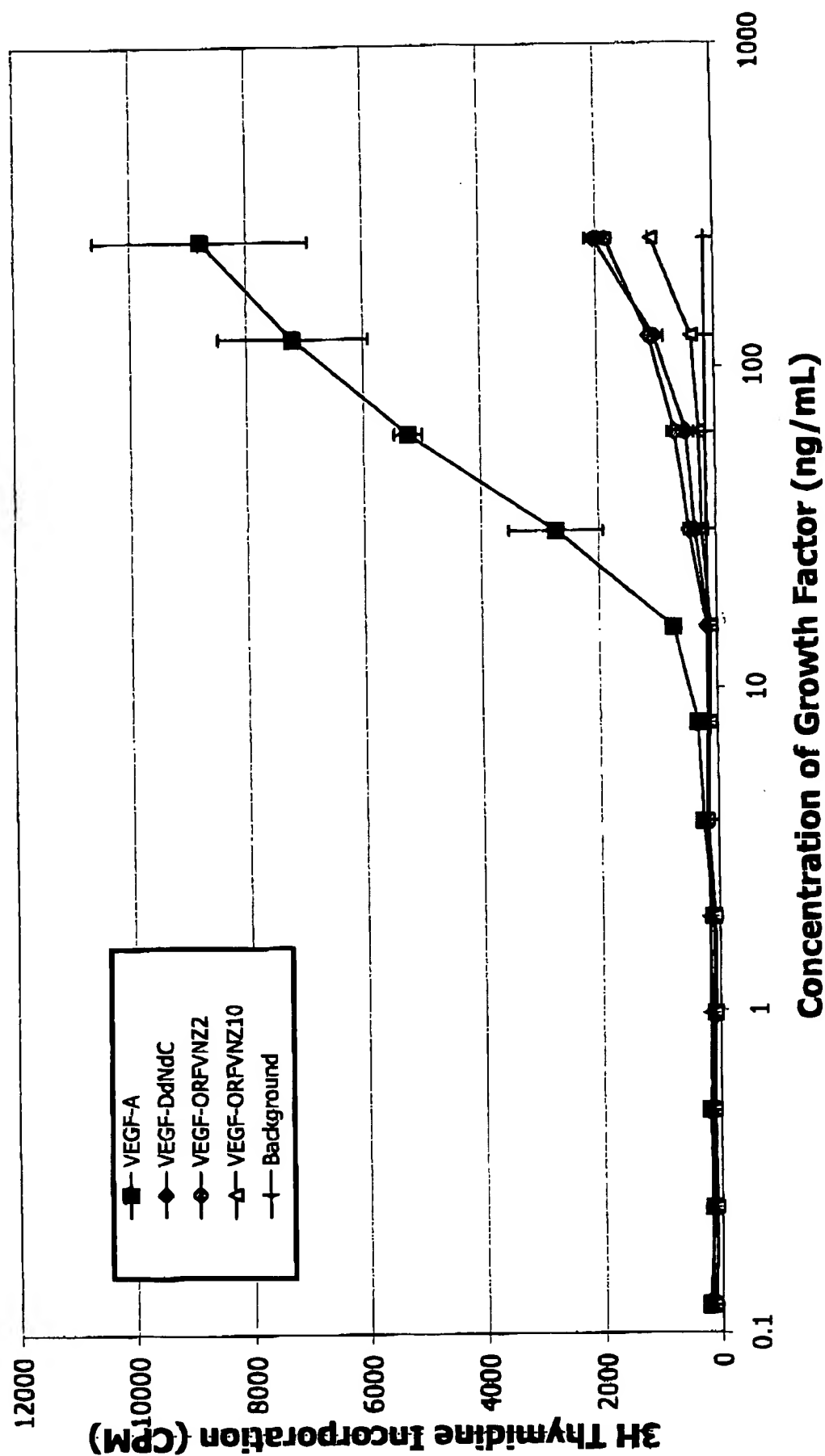
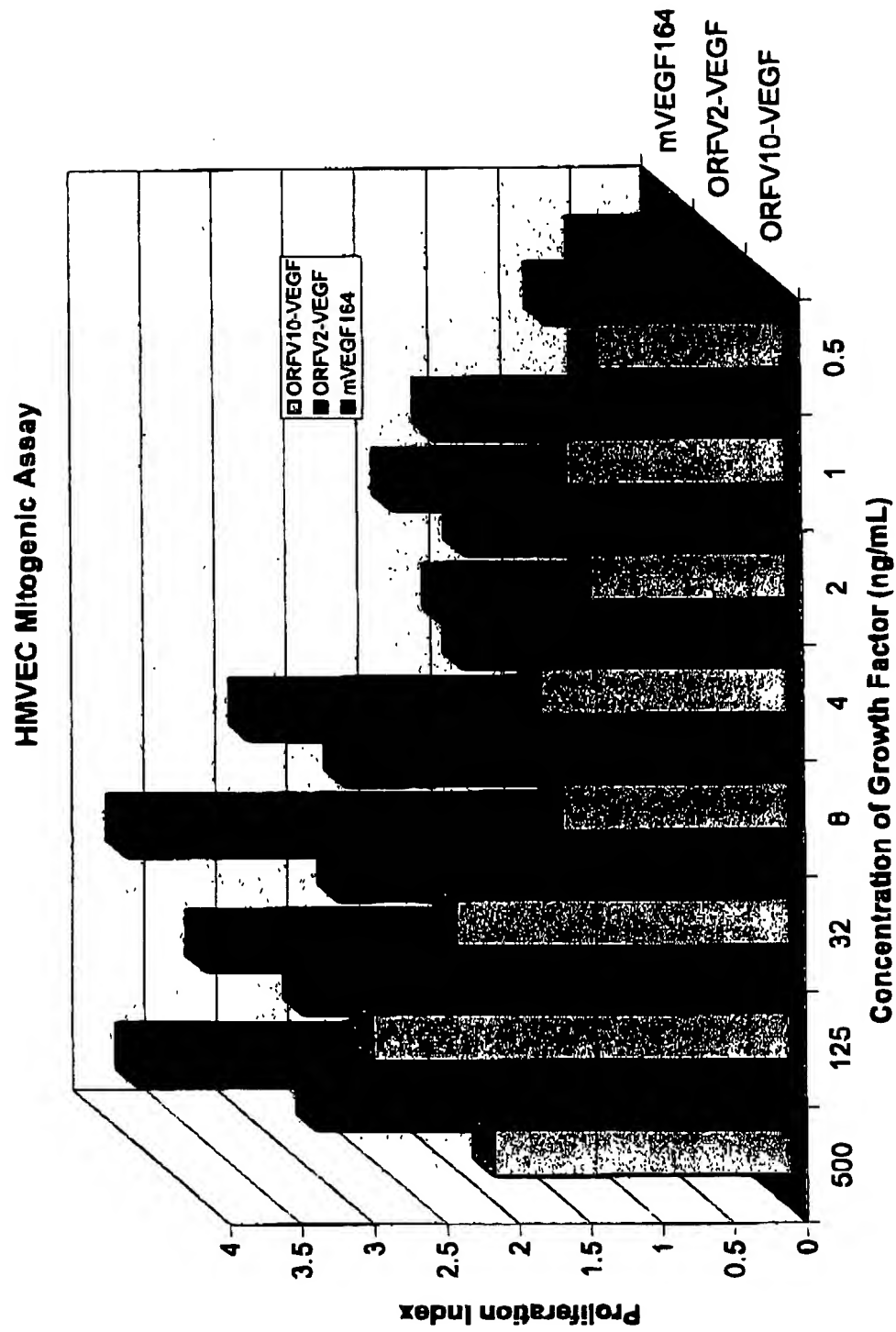


FIG. 3A

**FIG. 6A**

# HMVEC Mitogenic Assay

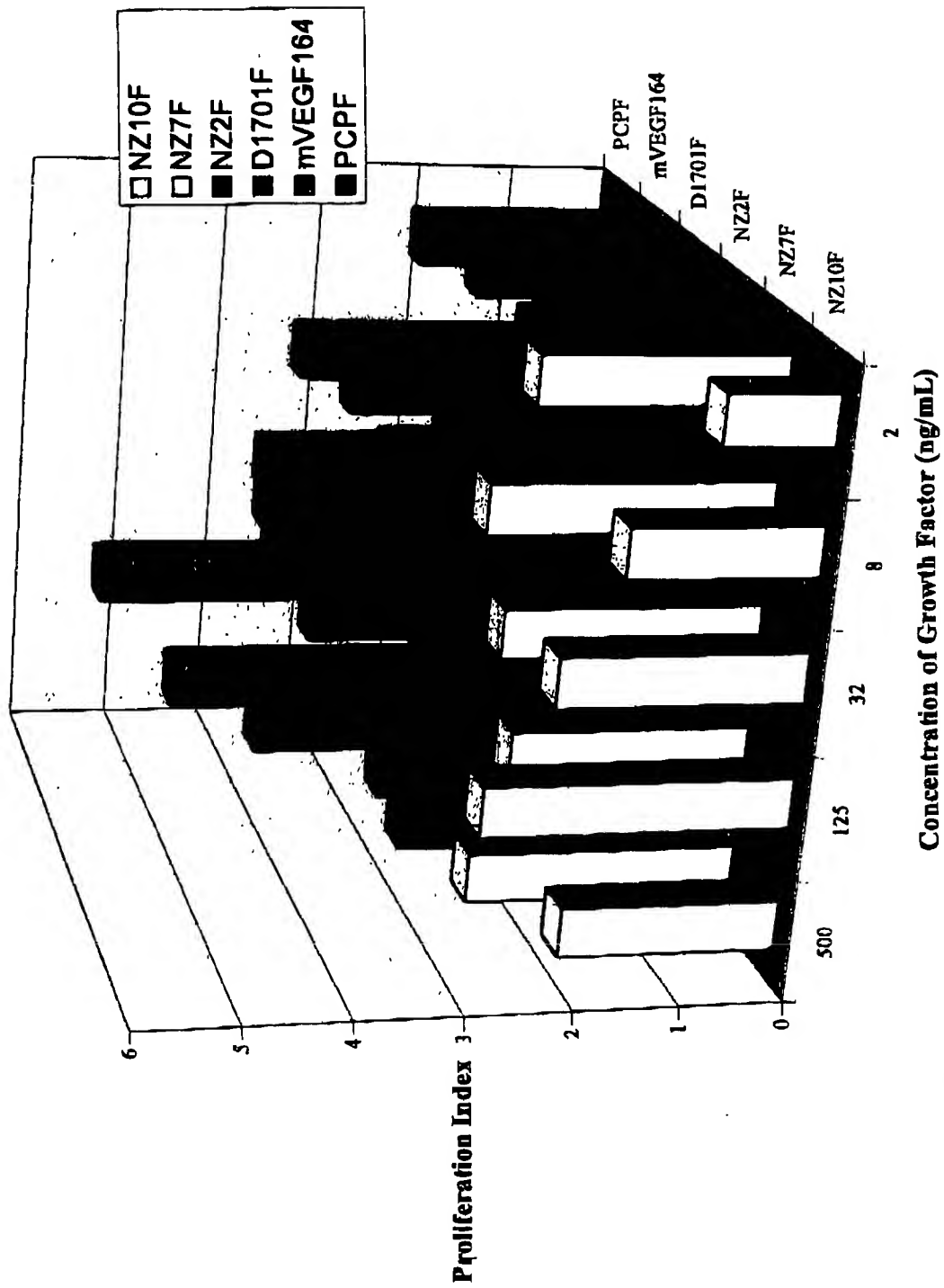


FIG. 6B